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Chemotherapy and Drug Targeting in the Treatment of Leishmaniasis

Annual Report

LINDA L. NOLAN

June 30, 1988

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-87-C-7146

UNIVERSITY OF MASSACHUSETTS
Amherst, Massachusetts 01003

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20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

Leishmaniasis, a disease caused by protozoan parasites of the Leishmania spp., is one of the major public health problems currently affecting humanity. Therapeutic agents for this disease is either ineffective or toxic. The purpose of this work is to aid in the development of an effective, non-toxic treatment for leishmaniasis.

The objective of this research was to isolate and characterize unique leishmanial enzymes (DNA polymerase) and to test promising antileishmanial compounds for toxicity against human CEM T_4 cells.

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MILITARY SIGNIFICANCE

The need for leishmanicides cannot be overemphasized. At present chemotherapy is dependent on a relatively small number of synthetic drugs. Resistance has been reported to occur against all these drugs and development of resistance to one compound is often accompanied by cross-resistance to others. In the chemotherapy of visceral and cutaneous leishmaniasis, the choice of drugs is very limited and success of a particular drug appears to vary from locality to locality, presumably due to strain differences in Leishmania.

To date the logical design of antiparasitic drugs has proved largely unsuccessful with the exception of purine metabolism in protozoa. While mammalian cells are capable of de novo synthesis of purines, many parasites do not synthesize purines but use salvage pathways. Analogues inhibiting key enzymes in purine pathway should, therefore, provide novel therapeutic agents. Purines and pyrimidines serve not only as precursors of RNA and DNA, but also as stores of high energy phosphate, constituents of certain coenzymes, and modulators of various enzymatic reactions. In view of this vital role, intervention of their metabolism will have profound effects on the organism.

To date there is no safe, effective, and quality-controlled antiparasitic vaccines. Membrane antigens differ from one species to another and during the course of infection, making the production of a useful vaccine very difficult.

The elucidation of the biochemical mode of action of promising compounds and the identification of unique enzyme systems will permit the logical design of more effective derivatives and also will provide insight on the mechanism of drug resistance. This information may allow a therapy program to be developed which would decrease or eliminate the problem of drug resistance.

Targeting of already promising compounds may increase the efficacy of these compounds for the various disease states of leishmaniasis and be more cost effective than the development of more than one drug.

Targeting will also allow the reduction in toxicity of certain compounds, and also be more cost effective since less drug should be required.

RESEARCH WITH T_4 CELLS

A hallmark of the Acquired Immune Deficiency Syndrome (AIDS) is the depletion and inactivation of CD4 T-cells. These are a specific type of T-lymphocyte that have the CD4, previously called T_4, cell marker on their surface membrane. They perform a number of functions that are essential to a normal immune response, and so are called "helper/inducer" lymphocytes. In response to antigen, they activate B cells to produce antibodies, stimulate cytotoxic T cells to attack foreign antigen, cause suppressor T cells to proliferate and thereby shut down an immune reaction when it is no longer needed, and stimulate macrophages to phagocytose and present foreign antigen. At the end of the immune response, CD4 T-cells proliferate into memory clones.

Any medication that is toxic to CD4 T-cells will seriously impair the patient's immune response and consequently worsen the prognosis of the patient. In the case of leishmaniasis patients, however, the toxicity of medications to CD4 T-cells is of crucial importance because the immune response must remain uncompromised for proper recovery from the disease. At this time, there has been no systematic assessment of the toxicity of medications used in the treatment of leishmaniasis to CD4 T-cells.

In the current study, we developed an assay to determine the toxicity of therapeutic agents to CD4 T-cells as measured by cell growth inhibition, and the reversibility of the toxicity by appropriate supplements. We then determined the toxicity of medications used in the treatment of leishmaniasis to CD4 T-cells, and developed a therapeutic index for each medication as an in vitro indication of the ratio of toxicity to host CD4 T-cells relative to the effective concentration of an agent i.e. the therapeutic value of that agent.

Leishmania. Thus by adding nutritional supplements which overcome metabolic pathway inhibition in human cells, but don't affect toxicity in Leishmania, a more effective therapeutic index can be obtained.

METHODOLOGY

A. Research Objectives:

The research objectives of this study are:

1) Develop an accurate assay to measure the toxicity of compounds on human T4 cells in vitro.
2) Determine the 50 percent inhibition level of purine analogs on human T4 cells in vitro.
3) Determine whether the inhibition of cellular growth by purine analogues can be reversed through the addition of specific natural compounds or nutrients.

B. Methods and Materials:

Cell Culture

CEM T4 cells were obtained from UMass Medical Center, Worcester. They are cultured in RPMI-1640 medium supplemented with 5% fetal calf serum, 1gm/liter Sodium Bicarbonate, and 1% gentamycin. In experiments, cells are grown in polystyrene microwell plates. The plates are incubated in a CO_2 chamber within an incubator at 36^0C. CO_2 prevents the growth medium from becoming too alkaline for cell survival.

Assay Procedure

The starting cell stock is standardized at an absorbance of 0.500, or a concentration of 2,600 to 2,800 cells/ul, because different concentrations of cells grow at varying rates, and are thus inhibited to variable extents. The cell stock concentration is standardized by centrifuging the cells down in a microcentrifuge for 3 minutes and resuspending in fresh medium to an absorbance of 0.500.

52 ml of the stirred cell stock are pipetted by multipipette into the plate wells, followed by 39 ml of double-concentrated, complete medium. To the control wells, 39 ml of sterile, double-distilled water is added. To test wells, increasing ratios of drug to sterile water is added. Four different concentrations of drug are used in an experiment, so that a curve of drug concentration versus cell growth inhibition can be determined from the results. The final volume of each well is 130 ml. A row of blank wells containing sterile water and medium only, is used to check the sterility of the procedure.

Cells are counted at the beginning of the experiment (time 0 hours) and after 120 hours of incubation, when the cells are in the logarithmic phase of growth. A Royco cell counter is used. 100 ml of each well's contents are diluted 100 times, and discharged into a vial. The vial is stirred and then measured by the cell counter. Percent inhibition is determined by:

[1- (test cell count / control cell count)] x 100.

Most purine analogs were supplied by the Walter Reed Army Institute, Washington D.C. Allopurinol Riboside was donated by Burroughs-Wellcome, North Carolina, and others were obtained from Sigma Chemical Co., St. Louis, MO.

Inhibition Reversal

Adenosine, adenine, and inosine will be the compounds first tested for inhibition reversal. In the case of sinefungin, the above three purines, as well as methionine, S-adenosylmethionine, and S-adenosylhomocysteine will be used in preliminary tests. Initially the toxicity of the reversal compounds themselves on CEM T4 cells will be determined by the above assay. In experiments, cells will be incubated with the reversal compound for 30 minutes in serum-free medium, before the purine analogue is added. Enzymes in the serum can metabolize the reversal compound before it enters the cell. In some cases, alterations in the effect of a purine analogue by a reversal compound only occurs if the compound is added first. Inhibition reversal will be determined by percent inhibition caused by purine analogue alone minus percent inhibition caused by purine analogue and reversal compound.

B. Operational Definitions

Toxicity: This refers specifically to the ability of a compound to inhibit the growth and reproduction of cells. Toxicity of a substance is generally proportional to its concentration. At relatively high concentrations, the toxicity of a substance kills all cells and so stops all growth completely. At relatively lower concentrations, partial cell growth occurs due to either inhibited growth or death of some cells affected by the compound.

Percent Inhibition: The inhibition of cellular growth caused by an agent as compared to undisturbed cellular growth of controls. It is measured by direct cell counts with a Royco or Coulter Cell Counter, and expressed as [1- (test cell count / control cell count)] x 100.

Fifty percent inhibition level: The concentration of an agent that inhibits cell growth by 50 percent, so that the cells grown with the agent have a cell count half that of the controls.

Assay: The assay is a means to measure the toxicity of compounds on cells. It involves growing cells in polystyrene microwell plates. Cells in control wells are grown in RPMI-1640 medium and water only. Cells in test wells are grown in medium, water and varying concentrations of purine analogs. Direct cell counts are made at time 0 and time 120 hours. The ratio of test well cell count to control well cell count is used as a measure of growth inhibition of toxicity of the purine analogs, and is expressed as percent inhibition.

Human CEM T4 Cells: This is the human T4-lymphoblastoid cell line, CCRF-CEM. They were originally derived from leukemic human T-helper-inducer (T4) lymphocytes, and are grown in culture.

Purine Analogs: These are compounds that have a chemical structure very similar to that of the purines adenine, guanine, and uridine which are utilized for the formation of DNA and RNA. Because of their structural similarity, they are mistaken for purines, get taken up in metabolic pathways leading to the synthesis of DNA, RNA, and proteins, and at some stage inhibit the normal production of these compounds. This in turn inhibits cellular growth and reproduction.

Nutrient additives for inhibition reversal: These are natural compounds found in food or food supplements that are involved in the metabolic pathways known or suspected to be inhibited by the purine analogues being tested. They include the regular purine nucleotide of which the test compound is an analogue. For example, the effects of allopurinol riboside can be reversed by adenine and related compounds (13), and Formycin A, and adenosine analogue, can be reversed by adenosine (14) in certain organisms. Adenosine, adenine, inosine and

L-methionine will be used in preliminary reversal studies. If the purine analogs is thought to inhibit an enzyme, the enzymatic product will possibly reverse the toxicity of the compound.

METHOD OF ANALYSIS

This study uses a classical experimental design, with one control and one experimental group. Before and after measurement of cell counts are done in both groups. a single-tailed, students' test is used to test for statistically significant differences between the control and test groups. At a significance level of 5% and a power of 85%, to prove a 10% difference between control and test group is statistically significant requires a sample of 24 controls and 12 tests.

The methodology fulfils the definitions of an experimental design by having: 1) a suitable control, 2) random assignment to control and test groups, and 3) manipulation of the independent variable i.e. purine analogs concentration. The controlled environment of the laboratory apparently eliminates many factors that could confound the results. For example, internal factors such as maturation and regression variables, as well as external factors such as selection, history, and experimental attrition variables are not the sources of concern they would be in a clinical trial. The main variables to control for in establishing causality are experimental factors such as accurate pipetting and cell counting, as well as maintaining sterile conditions.

SIGNIFICANCE OF THE STUDY

The first value of this study is in determining the toxicity on human-derived cells in vitro of several purine analogs. This is an indiction of their toxicity in living humans. These purine analogs have been reported in the literature or by private communication to exhibit powerful antiviral and antiprotozoal activity. Since the current treatment for leishmaniasis is inadequate (15,16), and our laboratory is working with Leishmania, the focus of this study is on the antileishmanial value of the purine analogs. Once the cytotoxicity of the compounds has been determined, their therapeutic index will be calculated by dividing leishmanial toxicity by CEM T4 cell growth inhibition. This will serve as an indication of the purine analogues' potential as chemotherapeutic agents for leishmaniasis.

The second part of the study will determine whether the therapeutic index of these potential antileishmanial agents can be increased by reversing the human cell growth inhibition with nutrient additives. Overcoming toxicity enhances the therapeutic value of the compounds, and adds to our knowledge of their site(s) of action, enabling synthesis of more effective compounds.

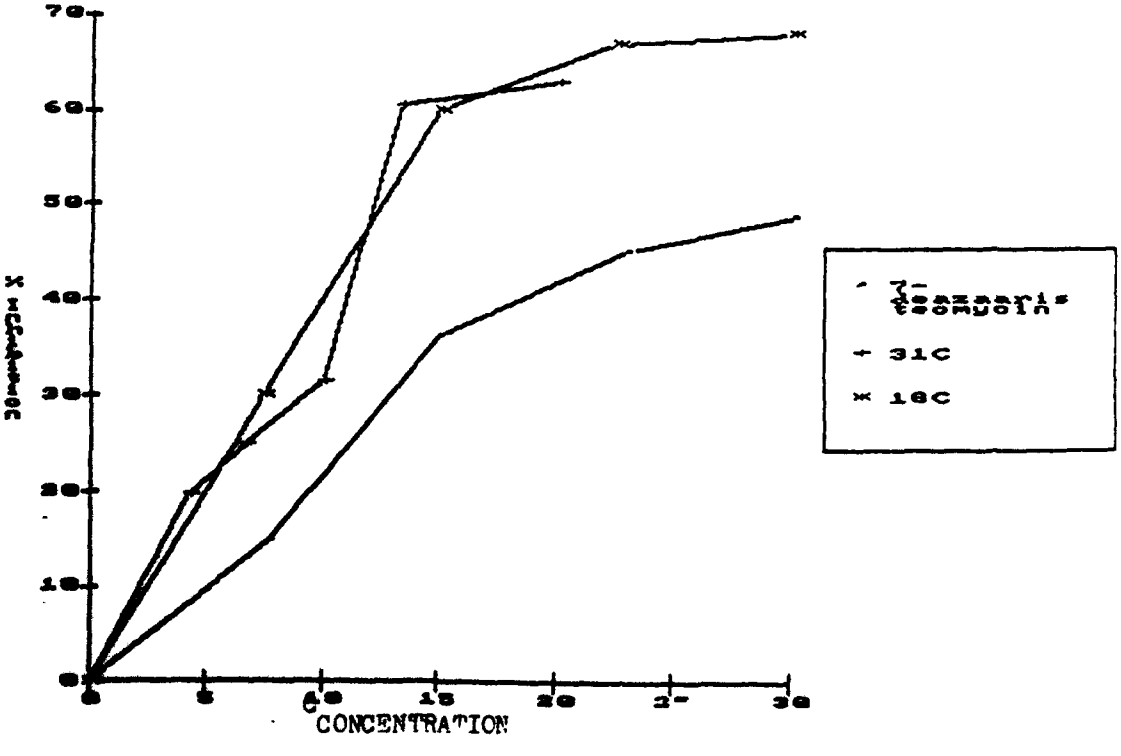
A second point of significance is that this study is an indication of the effect of potential chemotherapeutic agents on the host's immune system. The prognosis of leishmaniasis depends on the host's immune response (2). A simple case of cutaneous leishmaniasis can progress to an incurable, debilitating form in immunologically deficient persons (4). T4 lymphocytes are crucial elements in mounting an immune response to a foreign pathogen, as AIDS has proven. if a chemotherapeutic agent inhibits the growth of T4 cells, and thereby weakens the person's immune status, this will lead to an overall lower therapeutic value of the agent.

Tables 1 - 11 provide data on the ID_{50} of compounds tested in our CEM T4 cells. Table 12 summarizes the ID_{50} of all compounds tested.

SUMMARY OF DRUG INHIBITION.

DRUG ADDED.	CONCENTRATION of drug (uM)	% INHIBITION (1-test control *100)	EXPT #	ABSORB cell stock 660nm	TIME hours	FINAL CELL # per ml control
7-DEAZAARISTEOMYCIN	7.7 uM	14.8 %	40B	0.445	120hr	3,660
	15	36.1				
	23	45				
	30	48.6				
ID 50= +-33.4 uM.						
	4.2 uM	19.7%	31C	0.500	120	5,740
	6.7	24.9				
	10	31.4				
	13.3	60.4				
	20	61.9				
ID 50 = 12.4 uM .						
	7.7 uM	14.8%	40B	0.445	120hr	3,360
	15	36.1				
	23	45				
	30	48.6				
	7.5 uM	28%	24B	0.753 (17.3*1000/mm3	120	3,860
	11.5	42	"	"	"	
	52 uM	78%	15A	-	144	4,440
	98	77	15C	-	144	4,600
	7.5 uM	30	18C	0.521	120	4,400
	15	60	"	"	"	
	22.5	67	"	"	"	
	30	68	"	"	"	

ID 50 = 12.3 uM.



50% INHIBITION
40B = 33.4uM
31C = 12.4uM
18C = 12.3uM

TABLE 2

DRUG ADDED.	CONCENTRATION	% INHIBITION	EXPT #	ABSORBANCE	TIME hours	FINAL CELL #
9-DEAZAINOSINE	4.6 mM	19 %	43B	0.507	118hr	3,300
	9.2	38 %				
	13.8	46 %				
	3.1 mM	-2.1%	39A	0.507	118hr	4,900
	6.2	15				
	9.2	28				
	12	52				
	1.54 mM	-0.8%	38A	0.511	118	4,500
	3	4.0				
	4.6	8.1				
	6	12.5				
	.77 mM	3.9%	34B	0.491	120hr	3,980
	1.5	16.6				
	2.3	34.7				
	3.0	44.0				
	1.5 mM	14.3%	30B	0.500	117	3,870
	3.0	17.7				
	4.5	36.6				
	6.0	39.2				
	0.83 mM	5.0	21A	0.573	94	2,420
	1.04	15.8				
	1.25	18.4				
	1.50	28.6				
	0.19 mM	0	14E	-	115	3,600

50 % INHIBITION
In the range of 8-12 mM.

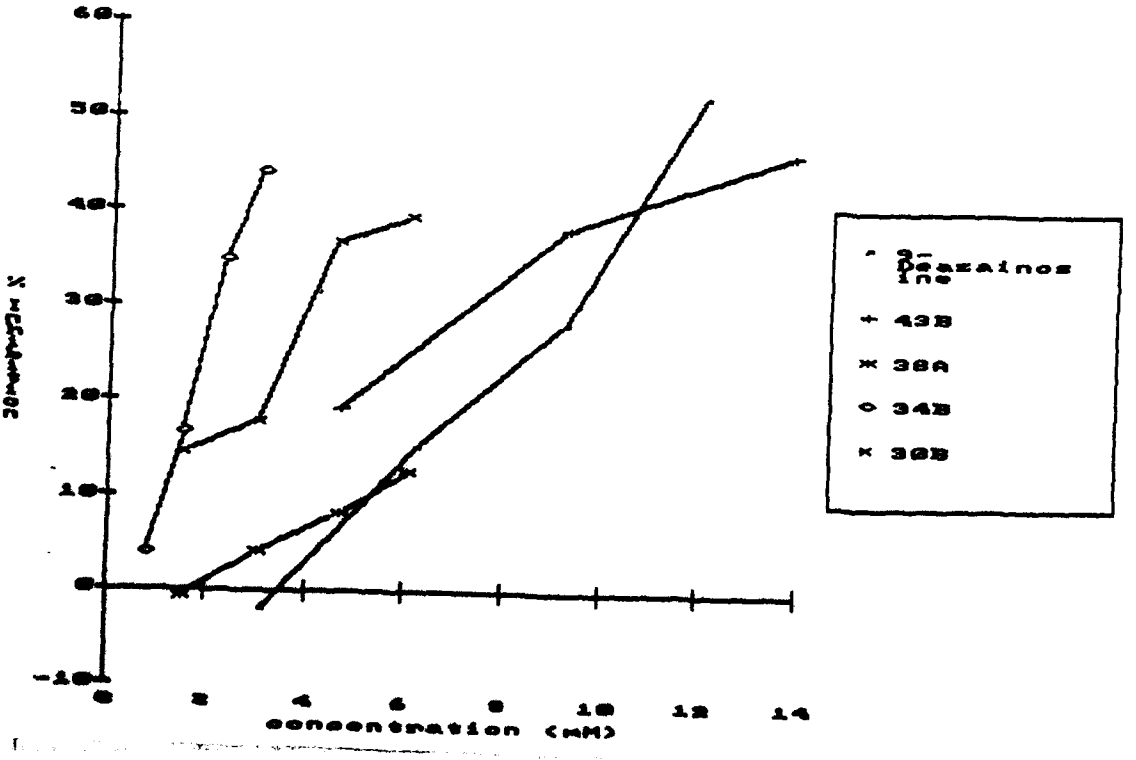
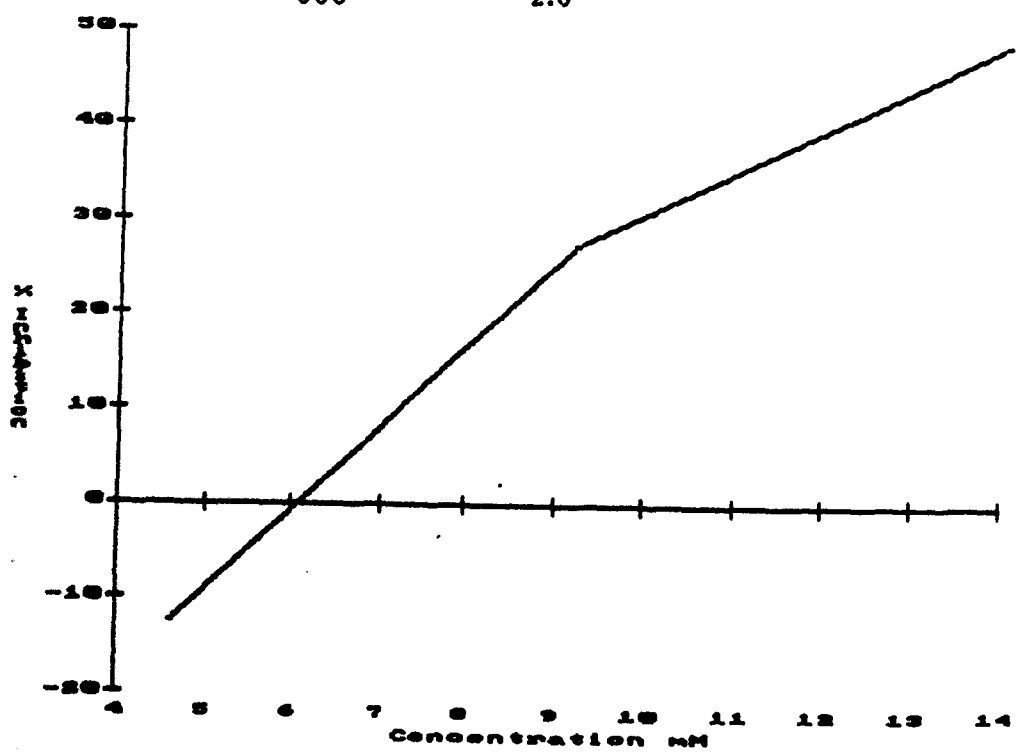


TABLE 3

DRUG ADDED.	CONCENTRATION	% INHIBITION	EXPT #	ABSORBANCE	TIME hours	FINAL CELL #
ALLOPURINOL-RIBOSIDE	4.6 mM	-12.6 %	43C	0.507	118hr	3,360
	9.2	27.1 %				
	14	48.4 %				
	3.1 mM	2 %	42B	0.480	119hr	3,540
	6.15	1.5 %				
	3.1 mM	-1.5%	39B	0.507	119hr	4,080
	6.15	3.4				
	9.23	11				
	12	19				
	1.4mM	-2.9%	38B	0.511	119hr	4,600
	3.0	-2.5				
	4.6	-3.3				
	6.0	-7.7				
	0.38 mM	-2.0%	34B	0.491	120	3,980
	0.77	0.5%				
	1.2	-5.5				
	1.5	-2.0				
	3.0	-18.6				
	83 uM	-.5%	30A	0.500	117	3,760
	167	5.3				
	250	8.0				
	600	0.5				
	83 uM	8.7%	29A	.500	127	4,400
	167	6.5				
	250	-1.0				
	600	-2.0				

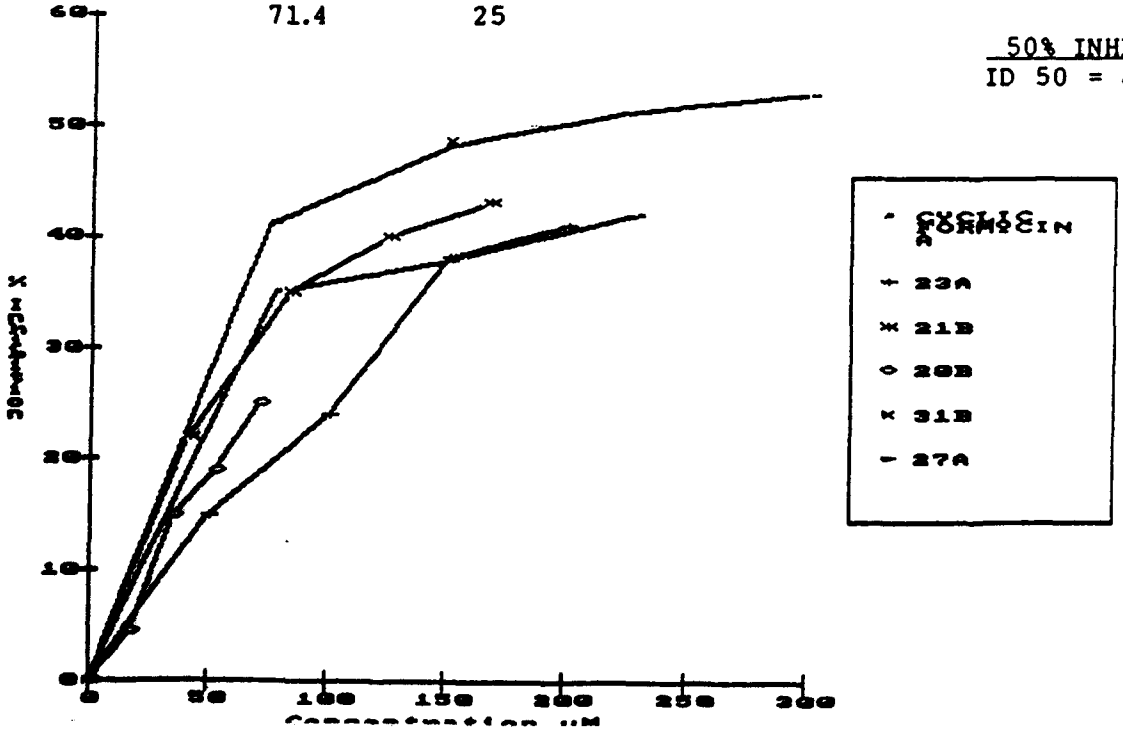


50 % INHIBITION
ID 50 = 14.3mM

TABLE 4

DRUG ADDED.	CONCENTRATION	% INHIBITION	EXPT #	ABSORBANCE	TIME hours	FINAL CELL #
CORDYCEPIN	150uM	-3%	19D	.773	144	6,940
	300	5				
	450	11.4				
	600	11.2				
	200uM	10%	15C	-	144	4,600
	103	7	15A	"	"	4,440
CYCLIC FORMYCIN A	77 uM	35.0%	45C	0.504	118hr	4,880
	154	38 %				
	230	42 %				
	300	39.3 %				
	150 uM	48.6 %	31B	0.500	120	4,500
	75 uM	41.2%	27A	2,860	142	5,150
	150	48.1				
	225	51.2				
	300	52.8				
	50 uM	15%	23A	0.538	96	3,200
	100	24				
	150	38				
	200	41				
	42 uM	22 %	21B	0.573	95	4,700
	83	35				
	125	40				
	167	43				
	17.8 uM	4.5 %	20B	-	120	3,500
	35.7	15				
	53.5	19				
	71.4	25				

50% INHIBITION
ID 50 = approx. 250uM



GRADE 5

DRUG ADDED.	CONCENTRATION	% INHIBITION	EXPT #	ABSORBANCE	TIME hours	FINAL CELL #
DFMO	1.9mg/ml	4.8%	45C	0.504	117hr	4,800
	4.8	40.7				
	7.5	63.7				
ID 50 = 5.9mg/ml						
	1.9mg/ml	4.9%	43D	0.507	118hr	3,100
	3.9	51.7				
	5.8	57.7				
	7.5	59.2				
ID 50 = 3.7 mg/ml						
	1.9 mg/ml	8.8%	39B	0.507	119hr	4,100
	3.9	10.8				
	5.8	19.6				
	7.5	29.0				
	1.54mg/ml	13%	34A	0.491	119	4,040
	3.1	42.6				
	4.6	46				
	6.0	57				
ID50=5.1 mg/ml						
	0.3 mg/ml	23.3 %	31C	0.500	120	5,740
	0.6	21.3				
	0.9	26.7				

50 % INHIBITION
45C= 5.9mg/ml
43D= 3.7mg/ml
34A= 5.1mg/ml

MEAN=4.9mg/ml

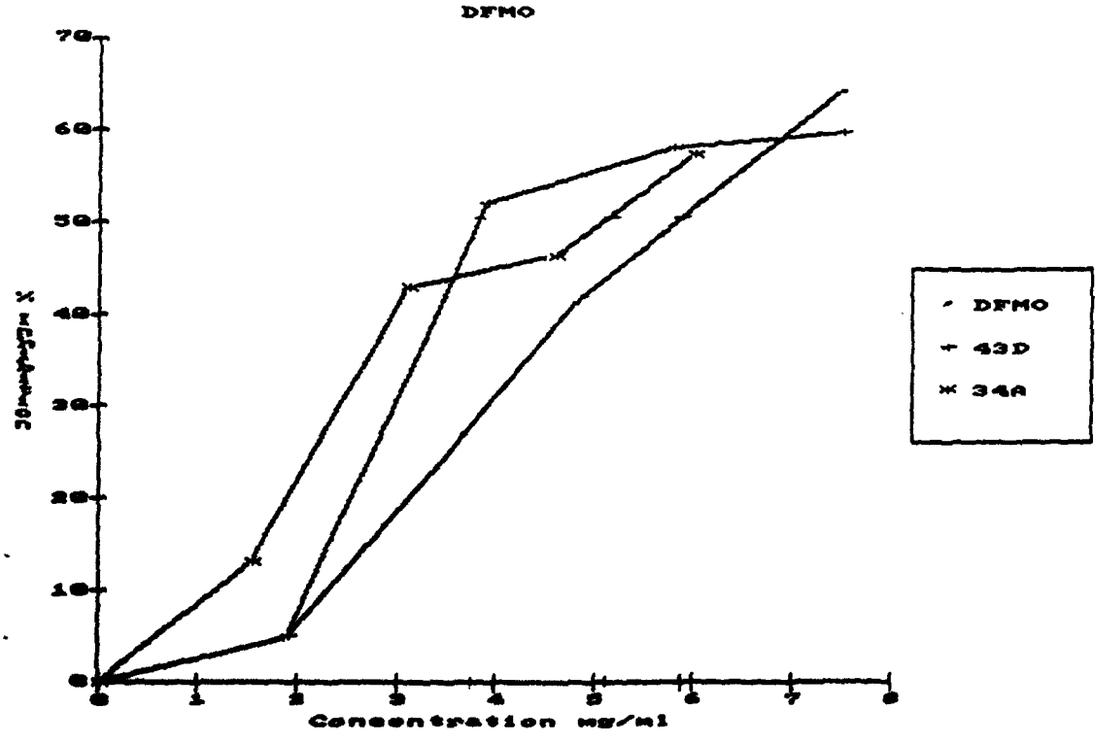
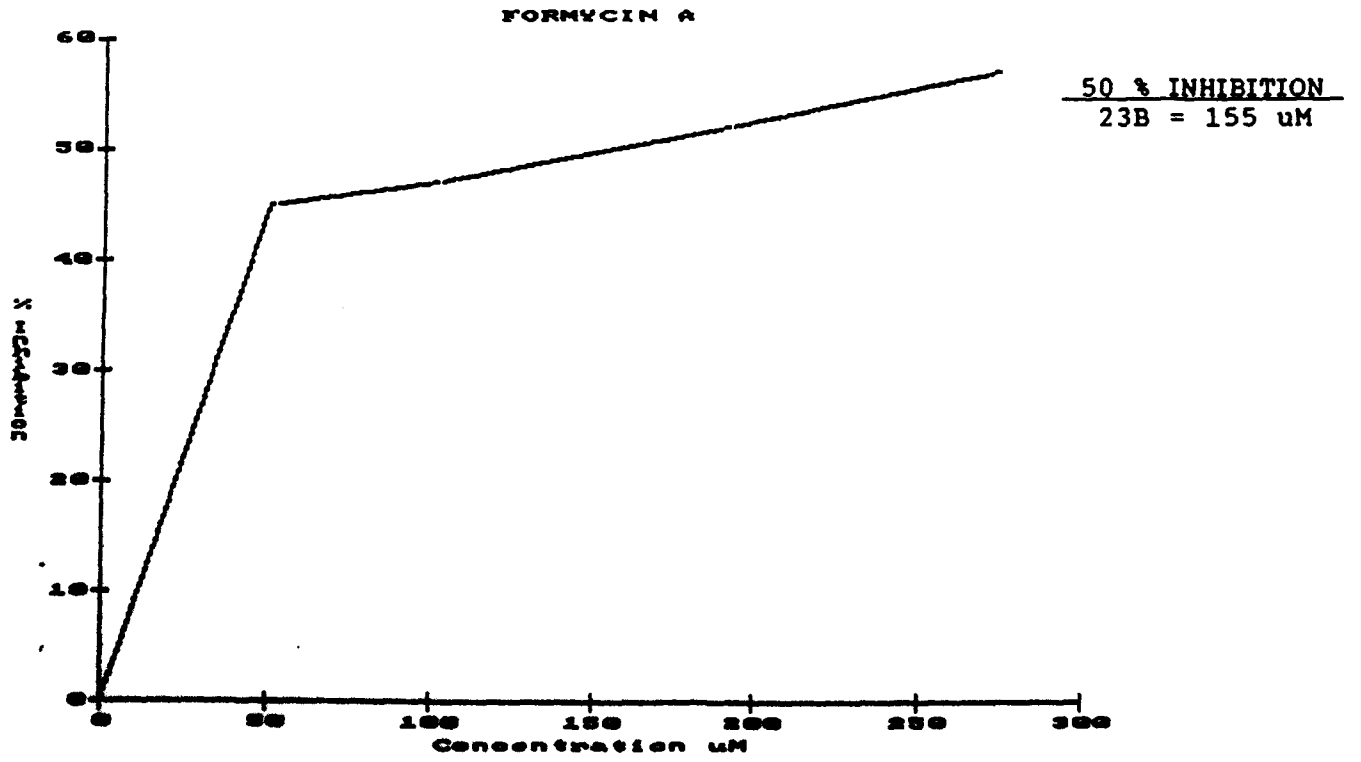


TABLE 6

DRUG ADDED.	CONCENTRATION	% INHIBITION	EXPT #	ABSORBANCE	TIME hours	FINAL CELL #
FORMYCIN A	50 uM	61.7 %	31A	0.500	120	4,540
	100	66.5				
	200	70.9				
	300	59.2				
	50 uM	45%	23B	0.538	96	17,000
	100	47	"	"	"	
	190	52	"	"	"	
	272	57	"	"	"	
FORMYCIN B	9.6 uM	60%	45B	0.504	118hr	2,780
	19.2	60.8%				
	29	50.4				
	37.5	54				
	19 uM	50.5%	43D	0.507	118	3,160
	39	51				
	58	51.4				
	100 uM	54.8 %	21B	0.573	95	4,700
	117	61.4				
	133	58				
	150	59				
	25 uM	52%	18B	0.521	120	5,020
	75	55				
	100	57				
	225	56				



50 % INHIBITION
23B = 155 uM

TABLE 7

DRUG ADDED.	CONCENTRATION	% INHIBITION	EXPT #	ABSORBANCE	TIME hours	FINAL CELL #
GLUCANTIME	920 ug/ml	-23%	45B	0.504	118	2,660
Lot # CA82-187-02	1,850	-4				
(STD-Glucitol)	3,600	16%				
	185 ug/ml	2.2%	43C	0.507	118	3,380
	370	1.0				
	555	-4.3				
	92 ug/ml	-6.6%	40B	0.445	120hr	3,660
	185	-13				
	277	-12.3				
	360	-20.8				
	46 ug/ml	-3.3%	38B	0.511	119hrs	4,600
	92	6.2				
	139	18.6				
	180	19.7				
Batch # 2	12.5	26	18C	0.521	120	20,200
	25	42	"	"	"	
	37	55	"	"	"	
	62	60	"	"	"	

50 % INHIBITION
Batch 1 = 32.4 ug/ml

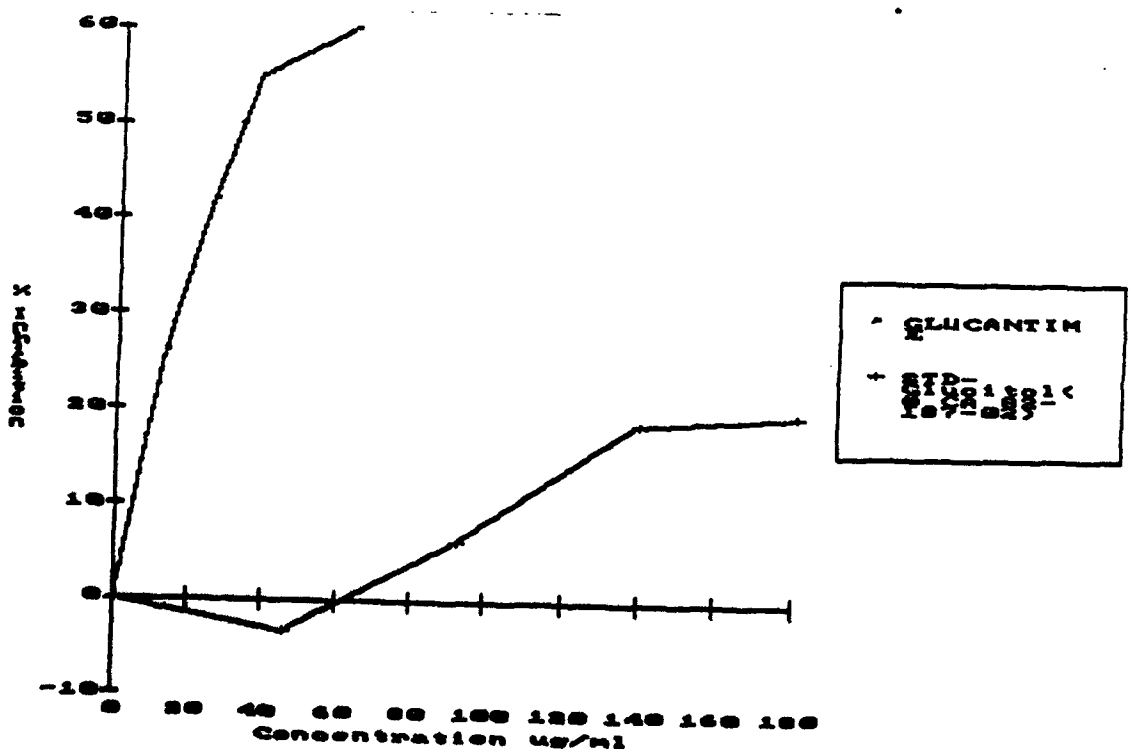


TABLE 8

DRUG ADDED.	CONCENTRATION	% INHIBITION	EXPT #	ABSORBANCE	TIME hours	FINAL CELL #
KETOCONAZOLE	1.56	54%	30A	0.500	118hr	3,760
	2.5	59.4				
	3.75	61.7				
	6.25	61.9				
	1.56 ug/ml	41.9%	29	.500	127	4,400
	2.5	47.2				
	3.75	55.6				
	6.25	62.9				
ID50=2.8 ug/ml.						
	6 ug/ml	66	28B	0.450	142	3,040
	11	69		(6,900)		
	17	71				
	22.5	68				
	3 ug/ml	45%	14A	-	115	4,200
	6	66				
	12	75				
	18	69				
	24	69				
	29	69				
ID 50 = 3.6 ug/ml.						
	15	82	12C	-	120	2,300
	30	85.4				
	60	90				
	90	87				
	120	97				
	150	86				

50 % INHIBITION
Expt 29 = 2.8 ug/ml
Expt 14A =3.6 ug/ml

Mean = 3.2 ug/ml

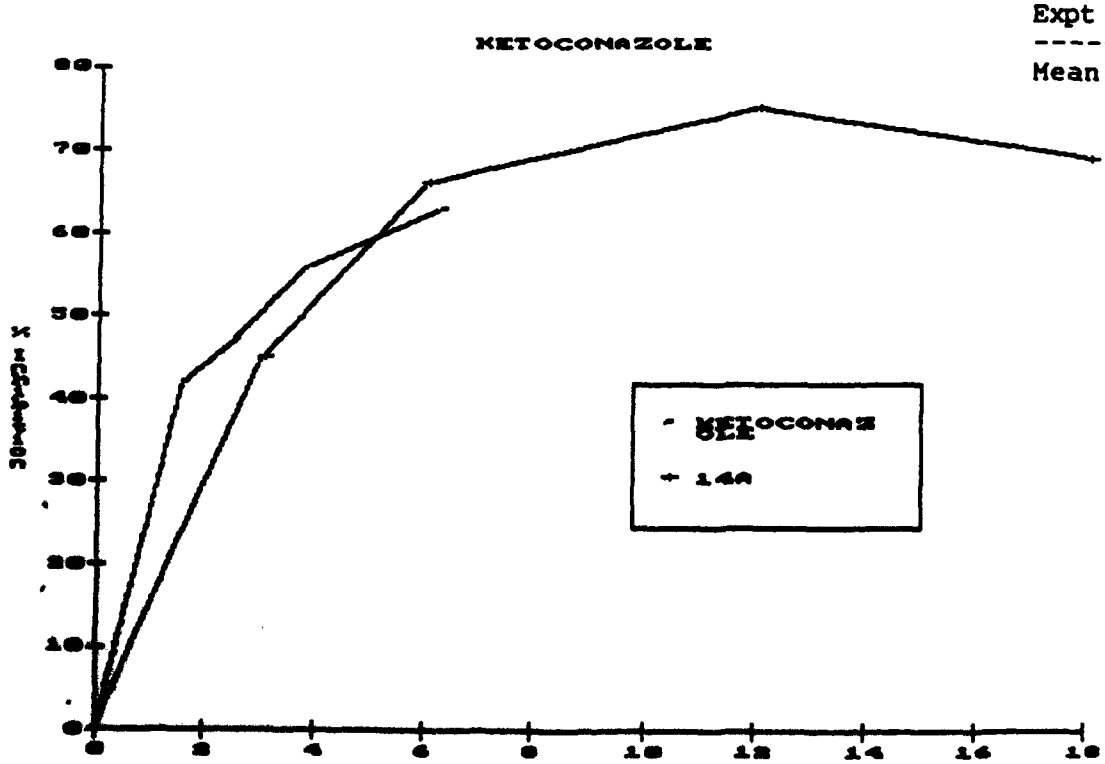


TABLE 9

DRUG ADDED.	CONCENTRATION	% INHIBITION	EXPT #	ABSORBANCE	TIME hours	FINAL CELL #
OXYFORMYCIN B	0.29 mM	15%	40A	0.445	119hr	3,450
	0.45	6.1				
	0.58	2.5				
	0.75	1.9				
	125 uM	21.2 %	31B	0.500	120	4,500
	250	22.1				
	458	17.1				
	667	28.3				
	125 uM	11%	23A	0.538	96	3,300
	250	11				
	375	3				
	500	10				
SANGIVAMYCIN	4 uM	47%	14E	-	115hr	3,600
	8	52				
	16	57				
	24	57				
	31	57				
	38	61				

50 % INHIBITION
ID 50 = 6.4 uM

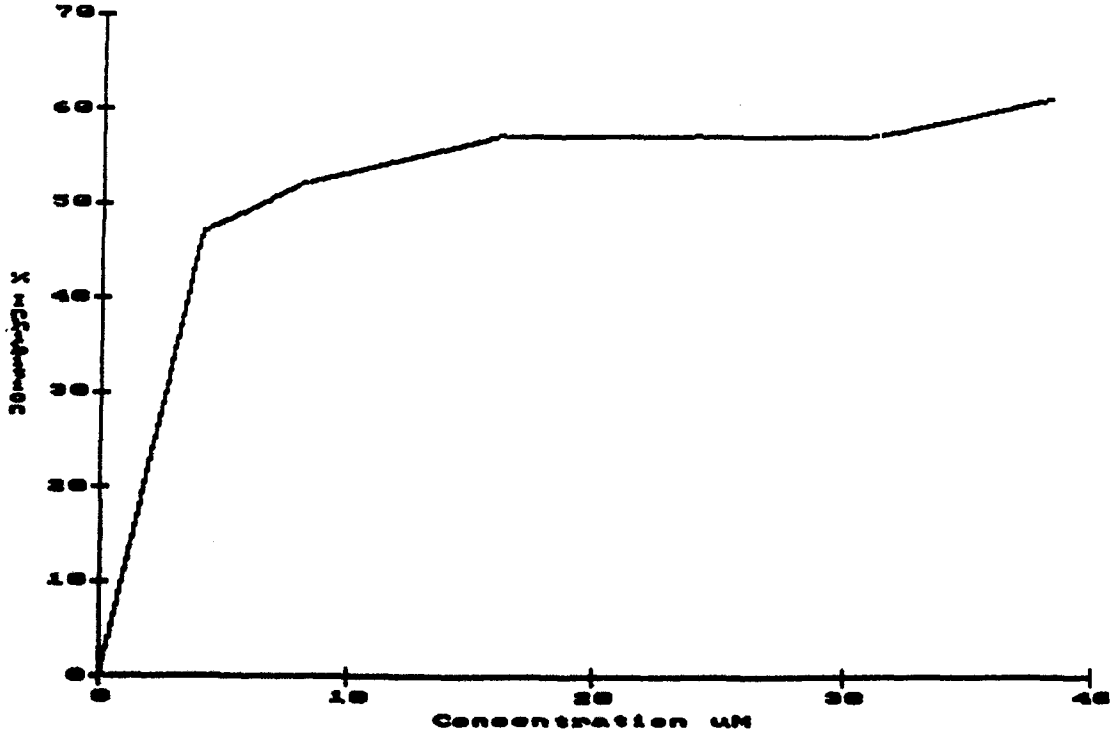


TABLE 10

DRUG ADDED.	CONCENTRATION	% INHIBITION	EXPT #	ABSORBANCE	TIME hours	FINAL CELL #
SIBA	75 uM	19%	19C	0.773	148hr	5,600
	150	44				
	225	47				
	300	51				
	107 uM	41%	16B	-	120hr	2,780
SPERMIDINE	83 uM	63%	21B	0.573	95	4,700
	35 uM	55%	20A	-	120	4,080
	120 uM	54 %	18A	0.521	120	3,540
	290	66	"	"	"	
	580	65	"	"	"	
	830	51	"	"	"	
	104	48.5	17	-	117	1,960
	229	52.3	"	"	"	
	280	51	16C	-	144	2,800
	540	58	16A	-	144	3,000

50 % INHIBITION
ID 50 =286 uM

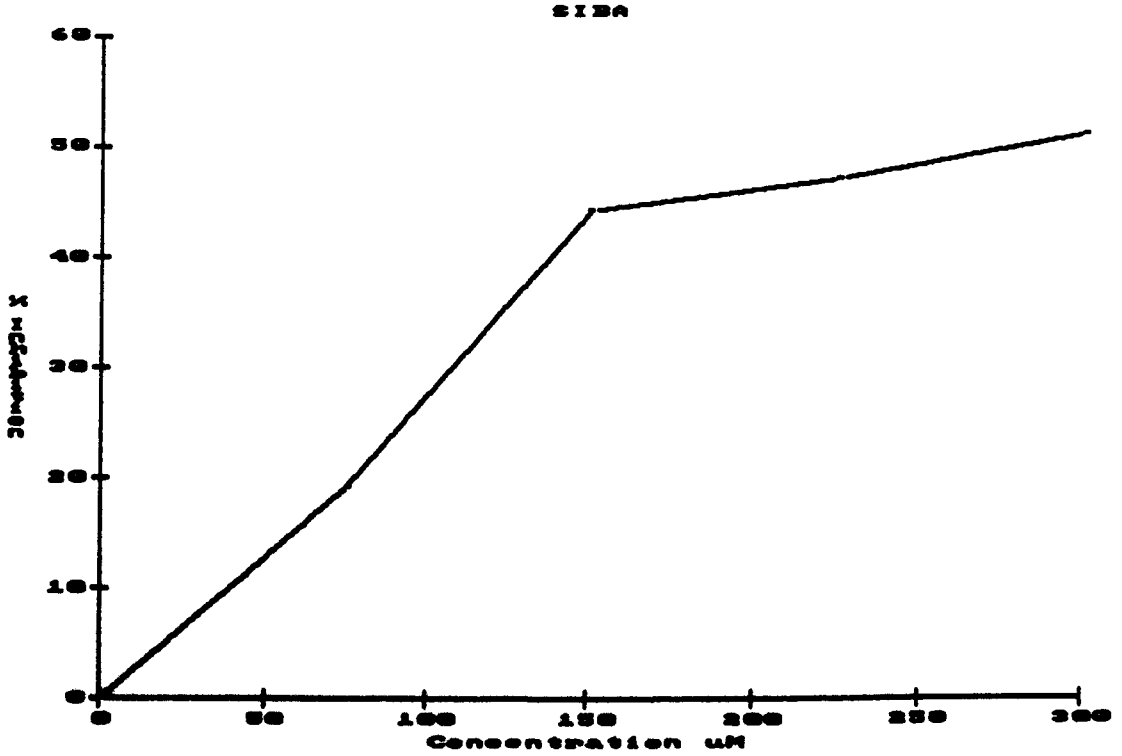


TABLE 11

DRUG ADDED.	CONCENTRATION	% INHIBITION	EXPT #	ABSORBANCE	TIME hours	FINAL CELL #
SINEFUNGIN	5.9 mM	37 %	43B	0.507	118hr	3,300
	11.8	42 %				
	17.7	37 %				
	23	36 %				
	3.1 mM	15 %	42B	0.480	118hr	3,400
	6.15	15.4 %				
	9.23	19.3 %				
	12	25.0 %				
	3.1 mM	23%	39A	0.507	118hr	4,840
	6.15	32				
	9.23	30				
	1.5mM	22.2%	38A	0.511	118hr	4,500
	3	21.7				
	4.6	29.3				
	0.77 mM	7.6%	34A	0.491	119	4,040
	1.54	9.0				
	2.31	9.5				
	248 uM	11.3 %	30B	0.500	117hr	3,870
	495	9.9				
	743	11.5				
	990	8.3				
	75 uM	9.1%	21A	0.573	94	2,420
	150	9.3				
	225	13				
	300	14.5				
	8 uM	0 %	14C	-	115	3,740
	16	8				
	32	3				
	48	7.7				
	63	14				

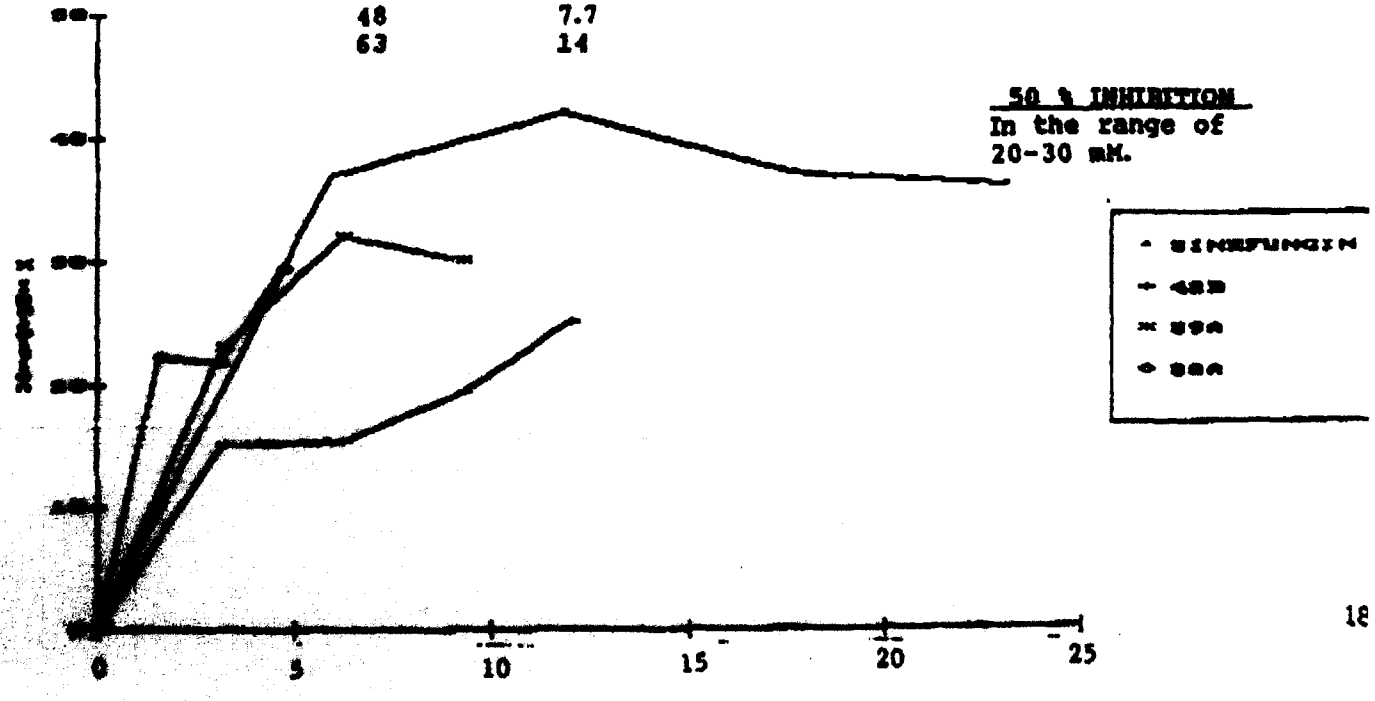


TABLE 12

Fifty Percent Inhibition Levels of The Purine Analogs Tested

Purine Analog	50 % Inhibition Level
7-Deazaaristeomycin	19.6 uM
9-Deazainosine	8-12 mM **
Allopurinol Riboside	14.3 mM **
Cyclic Formycin A	250 uM
DFMO	4.9 mg/ml
Formycin A	155 uM **
Glucantime (Lot #2)	32.4 ug/ml **
Ketoconazole	3.2 ug/ml
Oxyformycin B	0.8-2 mM *
Sangivamycin	6.4 uM **
SIBA	286 uM **
Spermidine	<100 uM
Sinefungin	20-30 mM *

* These ID 50s are extrapolated from inhibition curves.
** The reliability of these ID 50 values need to be verified by further experiments.

BACKGROUND - DNA POLYMERASE

Species of the parasitic protozoan genus Leishmania are the causative agents of a wide variety of human cutaneous, mucocutaneous, and visceral diseases. These organisms (lower eukaryotes) reside throughout their digenetic life cycles in different environments. The extracellular, flagellated promastigote forms reside in the alimentary tract of their sandfly vector hosts, and the obligate intracellular amastigote form exists within the phagolyso- somal system of macrophages in their mammalian hosts. How these organisms transform, survive, and respond to signals within their infected hosts is unknown.

Most of the major metabolic pathways in the parasitic protozoa have been reported to be similar to those of the mammalian host except for nucleic acid metabolism (41,43,44,73). This pathway is unusual in several ways. First, it lacks the ability to synthesize the purine de novo, making them entirely dependent on the salvage pathway for their supply of purine nucleotides. Second, many of the enzymes involved in nucleic acid biosynthesis either have unusual substrate specificities or unusual subcellular localizations (17-23,35,41-46,49,52-56,58-65,66-69). Third, a large proportion of the DNA which is produced is incorporated into a unique organelle known as the kinetoplast. Kinetoplast DNA, the mitochondrial DNA of leishmania and related parasitic protozoa, has a remarkable structure. It consists of networks, of thousands of interlocked DNA circles, and each cell has one network within its single mitochondrian (25,33,34,80). Nothing is known either about the function of kinetoplast minicircles or the reason that these molecules are interlocked, together with maxicircles, in an enormous network. Neither is it known why these parasitic protozoa, alone among eukaryotes, have their mitochondrial DNA organized in this unusual way. Fourth, the major DNA polymerase isolated from the parasitic protozoa has been shown to have different characteristics than its mammalian counterpart and to be immunologically distinct (28,71,72,81).

The presence of multiple DNA polymerases in eukaryotic cells is a well established fact. The use of specific inhibitors has helped to characterize nuclear and organelle DNA polymerases. DNA polymerase α involved in the replication of the nuclear genome is strongly inhibited by aphidicolin regardless of the source of the enzyme. Other eukaryotic DNA polymerases, like the β-polymerase involved in DNA repair as well as the chloroplastic and mitochondrial polymerases, are not affected by this drug (29,31,71,72,81).

Since the first description of a DNA polymerase in an animal cell 28 years ago (24), an immense body of information has been accumulated on eukarotic DNA polymerases, their classification, prevalence, evolution, physical and catalytic properties, and roles in DNA metabolism in vivo.

Animal cell DNA polymerases are distinctive from prokaryotic and viral polymerases and have been classified into alpha (α), beta (β), gamma (γ), and delta (δ) by cellular, physical, and enzymological parameters, as well as by their different responses to selective inhibitors (38).

Determining the roles of the four mammalian DNA polymerases — α, β, γ, and δ — is a fundamental problem in biology. Most studies conclude that

DNA polymerase α is the primary polymerase responsible for nuclear DNA replication. Recently, it was suggested by Byrnes that DNA polymerase δ may be involved in DNA replication. Polymerase δ is estabolised to contain intrinsic 3' to 5' exonuclease (proofreading) activity (26,27). Butylphenyldeoxyguanosine triphosphate (BuPdGTP) (58,85) and monoclonal antibodies directed against polymerase α have been shown to discriminate the activities of α and δ (27,30,70,84).

Chang et al. (1980) reported that extracts of bloodstream forms of Trypanosoma brucei showed that both DNA polymerase α and DNA polymerase β activities were present (29). The detection of DNA polymerase β in T. brucei demonstrated the presence of this enzyme in unicellular organisms. Chang also stated that DNA polymerase α was present in L. mexicana. They found the DNA polymerases in T. brucei to be immunologically distinct from host enzymes, and suggested that the structural differences between the parasite and the host enzymes could be exploited for the development of agents to combat parasitic diseases. Dube et al. (32) reported on the detection and characterization of DNA polymerase α in T. brucei and found that specific antisera that cross-reacted with mammalian DNA polymerase-α from different species failed to cross-react with the trypanosome polymerase (31,32).

Solari et al. reported that the surprising finding that Trypanosoma cruzi DNA polymerase (predominate form) failed to be inhibited by aphidicolin (81). Recently, Holmes et al. (1984) reported that a related organism Crithidia fasiculata had two types of DNA polymerase activity, the α-type reported as DNA polymerase A and a β-type reported as DNA polymerase B (49). The response of the C. fasiculata DNA polymerase A enzymes to inhibitors and utilization of poly(rA)-oligo (dT) showed these enzymes to be markedly different from mammalian DNA polymerase α. Aphidicolin had no effect on either the DNA polymerase A enzymes or on DNA polymerase B, at concentration of up to 250 μm. If their observations are correct, these lower eukaryotes will be truly unique in that their major mode of DNA replication is clearly different from that in mammals. The recent excitement about aphidicolin began with the demonstration that it is a specific, direct inhibitor of animal DNA polymerase α but is without effect on polymerases β or γ (51). Using aphidicolin as a tool, it has been shown that DNA polymerase α is clearly the principal polymerase required for DNA replication in all animals and plants studied.

Except for the brief report by Chang et al. (29), in which little experimental data was given, to our knowledge no one has characterized or performed kinetic studies with the DNA polymerases in Leishmania spp. We have begun studies to isolate the polymerases of L. mexicana for the purpose of elucidating key differences in DNA synthesis and its regulation by DNA and DNA polymerase binding proteins and other cellular modulators (i.e., hormones, prostaglandins, polyamines) between the parasitic protozoa and higher eukaryotes. This information will not only provide basic knowledge on evolution of DNA replication and regulation, but may provide information on how specific signals in the parasite's environment modify its morphology and biochemistry. The main purpose of the study is that elucidation of key differences between parasite and host will offer targets for chemotherapeutic exploitation.

In contrast to the findings of Holmes et al. (49), Solari et al. (81), and

Dube et al. (32), we have found that aphidicolin is inhibitory both to growth and to DNA polymerase (in vitro) of L. mexicana. We have found that inhibition is dependent on the purification of the enzyme. In crude preparations of DNA polymerase, aphidicolin (20 μM) inhibits over 50%, but as the enzyme is purified it is not inhibited at all or at 10-100X the concentrations used in crude preparations. Foster et al. (37) have reported that resistance of adenoviral DNA replication to aphidicolin is dependent on the 72-kilodalton DNA-binding protein. This protein protects the DNA polymerase of the virus from inhibition by aphidicolin. However, this protein does not appear to protect host cells infected with the virus from inhibition by aphidicolin (37). The possibility exists that in vitro a similar type protein protects the DNA polymerase of parasitic protozoa. Although aphidicolin has been shown to inhibit DNA synthesis (of parasitic protozoa) in vivo more efficiently than ethidium bromide and berenil, this laboratory has been the first to demonstrate inhibition of DNA polymerase in vitro under certain conditions.

Binding of proteins to DNA and DNA polymerase is fundamental to the mechanism of the control of gene expression in both prokaryotic and eukaryotic cells (83). Knowledge of the specific molecular features of DNA recognized by complementary features of the three-dimensional structure of the DNA-binding proteins is still in its infancy. The binding proteins form stoichiometric complexes which modulate subsequent enzymatic transformations.

Prokaryotic binding proteins have been shown to be essential in initiation and elongation in DNA replication (39,50) and in DNA repair and recombination (16). Binding proteins isolated from calf thymus (47,48), Ustilago maydis (17), mouse ascites cells (75), and other eukaryotic sources have been shown to stimulate DNA polymerase activity. There is no information on the characteristics and function of DNA-binding proteins in Leishmania sp. or other parasitic protozoa.

SPECIFIC AIMS

The overall aim of this contract is to search for differences which exist between the DNA synthetic machinery of the lower eukaryotic parasitic protozoa Leishmania mexicana and mammalian cells for the purpose of studying the evolutionary development of the DNA polymerases for the purpose of chemotherapeutic exploitation. The differences in the enzymes are being characterized by cellular, physical, and enzymological parameters, as well as by their different responses to selective inhibitors and microbial excretory products shown to inhibit DNA synthesis in vivo and in vitro.

A. DNA polymerase(s) ($\alpha, \beta, \gamma, \delta$) are being isolated from L. mexicana and T. cruzi. Their characteristics will be compared to each other and to mammalian DNA polymerases from Chinese hamster ovary cells.

   Inhibitors are being sought which preferentially target the DNA polymerase of the parasite. Particular emphasis is being placed on purine analogs which are uniquely metabolized inside the parasite (taking advantage of enzymes which metabolize allopurinol and Formycin B as an example [73] or purine analogs which have been shown to be

much more toxic to parasitic protozoa than to mammalian cells (i.e., sinefungin) (11,76).

B. At each step of purification not only are the DNA polymerase(s) being monitored, but also other enzymatic activities associated with DNA polymerase complex formations and activity. The following enzyme assays are being performed:

Nuclease activity (DNA and RNA)
Nucleoside diphosphokinase
endo DNAase
exo DNAase
ATPase
Pyrophosphatase

The effect these enzymes have on polymerase activity and product formation are being monitored by a radioisotope enzyme assay by electrophoresis and subsequent densitometry.

C. DNA binding proteins are being isolated and added to the DNA polymerase during different stages of purification and in the presence of varying conditions (i.e., templates, metals, inhibitors) to determine how they modify DNA polymerase activity. Synthesis of binding proteins (found to alter DNA polymerase) in both promastigotes and amastigotes of L. mexicana are being studied.

D. Mutants of Leishmania mexicana to both aphidicolin and sinefungin will be obtained to elucidate their mode of action.

E. The drug mutants, which will be the promastigote form, will be grown in a macrophage system to determine if they are able to transform to the amastigote form.

F. The mode of action of natural products such as arachidonic acid; linoleic; and sinefungin, aphidicolin, and Clostridium perfringens enterotoxin (microbial excretory products) on DNA polymerase will be determined.

SIGNIFICANCE

There is very little information on the DNA polymerases, and the DNA-binding proteins in the parasitic protozoa of the family Trypanosomatidae. The information available on the DNA polymerases of these lower eukaryotes has indicated that these enzymes are biochemically and immunologically distinct from mammalian DNA polymerases. Information on the characteristics of the DNA polymerase of these lower eukaryotes and how binding proteins affect replication will provide insight on the evolution of the DNA replicatory machinery. These lower eukaryotes serve as an excellent model since they are masters at evading the immune response and biochemically and immunologically are able to change forms in their insect vector and mammalian hosts. The effect of changes and mutations in the DNA polymerase and binding-proteins can be studied through the process of transformation from the insect form to the infective mammalian form in a macrophage cell line.

In contrast to the DNA polymerase of higher eukaryotes the DNA polymerase α-like (when purified over 4000-fold) from parasitic protozoa is resistant to inhibition by aphidicolin, yet aphidicolin is a potent DNA synthesis inhibitor *in vivo*. Using aphidicolin as a tool we can investigate the differences in the structure of DNA polymerase of lower and higher eukaryotes and the influence and function of binding proteins.

The need for leishmanicides cannot be overemphasized. Twelve genera of parasitic protozoa are known to cause infection in mammals. There exist four major species of pathogenic *Leishmania*: (1) *L. donovani*, which causes visceral leishmaniasis and is often fatal; (2) *L. braziliensis*, the agent for new world form of cutaneous leishmaniasis; (3) *L. mexicana*, which causes the new world form of cutaneous leishmaniasis; and (4) *L. tropica*, which produces old world cutaneous leishmaniasis. At present, chemotherapy is dependent on a relatively small number of synthetic drugs. Resistance has been reported to occur against all these drugs and development of resistance to one compound is often accompanied by cross-resistance to others. In the chemotherapy of visceral and cutaneous leishmaniasis, the choice of drugs is very limited and success of a particular drug appears to vary from locality to locality, presumably due to strain difference in *Leishmania*.

A comparison of the enzymes of the pathogenic protozoa to those of man is of fundamental importance to the search for much needed chemotherapeutic agents. Nucleic acid metabolism in trypanosomatids is unique in several ways: (1) they lack the ability to synthesize purines *de novo*, depending entirely on the salvage pathway for their supply of purine nucleotides; (2) many of the enzymes involved in nucleic acid biosynthesis either have unusual substrate specificities or unusual subcellular localizations; (3) a large proportion of the DNA which is produced is incorporated into a unique organelle known as the kinetoplast; and (4) the DNA polymerase isolated from these organisms demonstrates major differences from its mammalian counterpart.

Detailed knowledge of the DNA polymerase(s) of parasitic protozoa will provide information for the further design of more effective drugs and provide insight into drug resistance. To date there is no safe, effective, and quality-controlled antiparasitic vaccine. Membrane antigens differ from one species to another and during the course of infection, making the production of a useful vaccine very difficult. Until an effective vaccine is produced, treatment of leishmaniasis will have to be through chemotherapy.

Not only will information gained from such studies be useful in chemotherapeutic exploitation of parasitic protozoan enzymes, which differ from host cells, but may help elucidate the control mechanisms necessary for normal DNA synthesis in higher and lower eukaryotic animal cells and help us recognize abnormal characteristics.

Also, the effect of natural products on the DNA polymerases will be studied for the purpose of exploring regulation of the DNA polymerases and for the purpose of studying how prokaryotes and lower and higher eukaryotes developed evolutionally to compete in their environments against each other. These studies will help elucidate major differences between parasite and host that uncover chemotherapeutic targets.

PROGRESS

Partial Purification of DNA Polymerase from L. mexicana

A. The major DNA polymerase(s) of L. mexicana were purified 4,000-7,000-fold (Tables 13 and 14).

B. The polymerase(s) were characterized with the use of inhibitors, salt, pH, heat, metal requirements, and specific inhibitors. One enzyme was found to resemble mammalian DNA polymerase α but had many differences. Response to inhibitors is shown in Table 15.

C. Aphidicolin, a known DNA polymerase α, did not inhibit the purified enzyme, but inhibited polymerase activity in the crude state and in protozoan cells in vivo) (Figure 1).
Sinefungin, a promising antiparasitic agent, inhibited the polymerase during the early steps of purification, but not in the latter steps. Sinefungin had a ki 15 nM, and inhibition was completely reversed by dATP (Figures 2 and 3). Both aphidicolin and sinefungin are potent inhibitors of leishmanial growth (Figure 4). S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) closely related compounds to sinefungin (Figure 5) had no effect on the DNA polymerase.

D. Using the method of Holmes et al. (49), DNA polymerase β was separated from and partially purified, utilizing chromatography an DEAE 23, P11 phosphocellulose, and Sephacryl S-300 (Table 16, Figure 6).

E. Arachidonic acid, a precursor to the prostaglandins and modulator of the immune response (35,57,74,77,78,82) was found to be both a potent growth inhibitor of promastigotes (50% Inhibition = 0.5 μM) and a potent DNA polymerase inhibitor (50% Inhibition = 16 μM) (Figure 7) Several recent publications (35,57,74,77,78,82) have demonstrated that arachidonic acid metabolism is altered in infected macrophages and that because of this alteration the normal immune response is suppressed. Inhibitors blocking arachidonic acid metabolism during infection help restore immune function (79). Perhaps release of arachidonic acid by the action of phospholipase A during infection is an attempt by the host to kill its invader. If the invader then metabolizes the released arachidonic acid to prostaglandins by its own enzymes and suppresses the immune response, it has successfully evaded its host.

F. It was demonstrated that purified Clostridium perfringens Type A enterotoxin (one of the major causes of food poisoning in humans) was extremely inhibitory to leishmanial DNA polymerase α-like and to DNA polymerase from Chinese hamster ovary cells (CHO) (Figure 8). These studies were performed in collaboration with Dr. Ronald Labbe, an expert on C. perfringens enterotoxin (University of Massachusetts, Amherst), and Dr. Nasseema Khan, an expert on DNA polymerase α from CHO cells. Our laboratory is the first to demonstrate the inhibitory response of a microbial enterotoxin on lower and higher eukarotic DNA polymerases. The toxin was isolated by the method of Granum et al. (40).

Table 13.
Purification Scheme for DNA Polymerase

Purification Step	Total Volume (ml)	Total Protein (mg)	Units[a] $(ml)^{-1}$	Total Activity (units)	Specific Activity (units mg^{-1})	Purification (-fold)
Lysed cells	40.8	1448.4	6.70	273.18	0.189	
Crude extract (15,000 rpm, 1 hr)	38.5	1068.38	7.7	296.45	0.277	1.47
Protamine Sulfate Supernatant	37.5	441.0	15.29	573.36	1.3	6.88
Heparin-Sepharose	14.5	41.62	119.77	1736.67	41.73	220.79
Cellulose Phosphate	10.2	4.38	26.58	271.14	61.82	327
DNA Cellulose	3.1	0.108	29.08	90.15	834.72	4416.51

[a]One unit of activity is defined as the incorporation of 1 pmole of dTMP into DNA in 30 min under standard assay conditions. Assay conditions are described in Methods.

Purification of DNA Polymerase from Leishmania mexicana 227

Purification Step	Total Vol.(mls)	Total Protein(mg)	Units/ml	Specific Activity (Units/mg)	Purification Fold
Crude Extract	20.0	3,186.60	2.66	0.016	--
S-100 fraction	18.00	1,656	1,72	0.019	1.17
DEAE Cellulose	36.00	56.06	1.55	0.099	6.24
Phospho-cellulose	6.80	29.71	32.27	7.38	461.57
DNA Agarose Peak #1	1.25	1.925	7.83	5.08	317.73
DNA Agarose Peak #2	1.25	0.331	32.56	122.96	7,685

Table 14

Table 15 DNA Polymerase α-like Inhibition

Drug	Minimum Concentration (Inhibition)	Maximum Concentration Tested	Minimum Concentration Giving 100% Inhibition
Novobiocin	0.5 µM (5.7%)	5.0 µM (83%)	10.0 µM
Ethidium Bromide	0.5 µM (31.4%)	1.5 µM (38.18%)	2.5 µM
Nalidixic Acid	0.5 µM (5.8%)	2.25 µM (37.13%)	5.0 µM
Phosphomycin	5.0 µM (7.1%)	50.0 µM (65.90%)	-*
Phosphonoacetic Acid	5.0 µM (8.5%)	38.0 µM (35.56%)	-
Mitomycin C	0.4 µM (31.64%)	0.5 µM (84.80%)	1.0 µM
N-Ethylmaleimide	-	5.0 mM (84.79%)	10.0 mM**
Coumermycin A	5.0 µM (2.80%)	25.0 µM (2%)	-
Aphidicolin	5.0 µM (1.42%)	26.6 µM (78.60%)	-
Berenil	0.5 µM (18%)	2.0 µM (58.0%)	3.5 µM
Cytosine-β-D-Arabinofuranoside	100.0 µM (24.36%)	200.0 µM (34.94%)	-
Cytosine-5-carboxylic Acid	100.0 µM (41.94%)	200.0 µM (50.76%)	-
2',3'-Dideoxyadenosine-5'-triphosphate	100.0 µM (12.04%)		-
Dideoxythymidine-triphosphate	100.0 µM (43%)		
Dideoxycytidine-triphosphate	100.0 µM (71%)		

*Symbol for has not been determined.

**97%

Figure 1. Growth Inhibition of L. mexicana by Aphidicolin

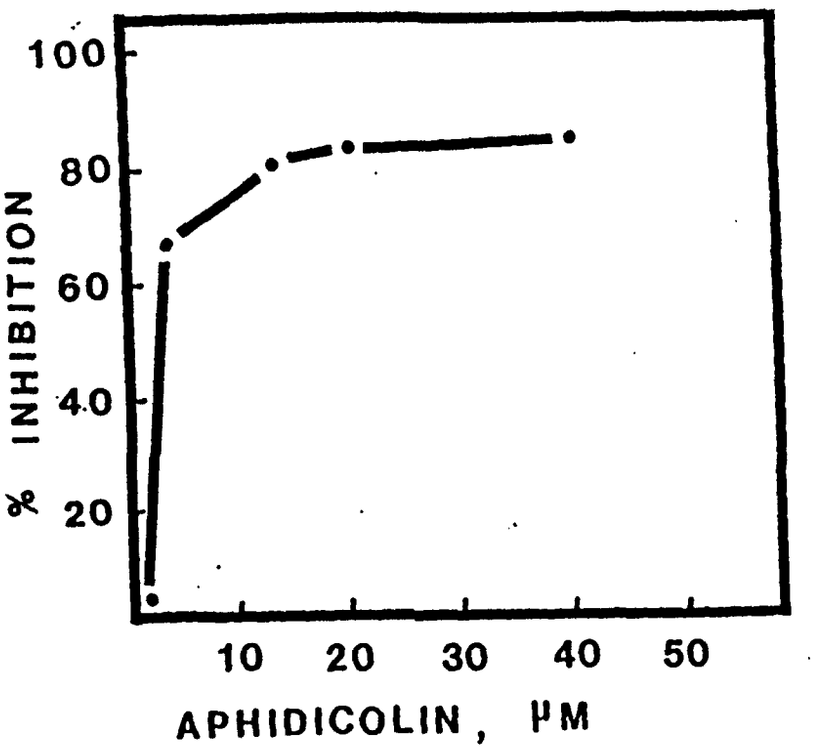


Fig. 2. Dixon Plot of Increasing Concentrations of Sinefungin in the Presence of dATP.

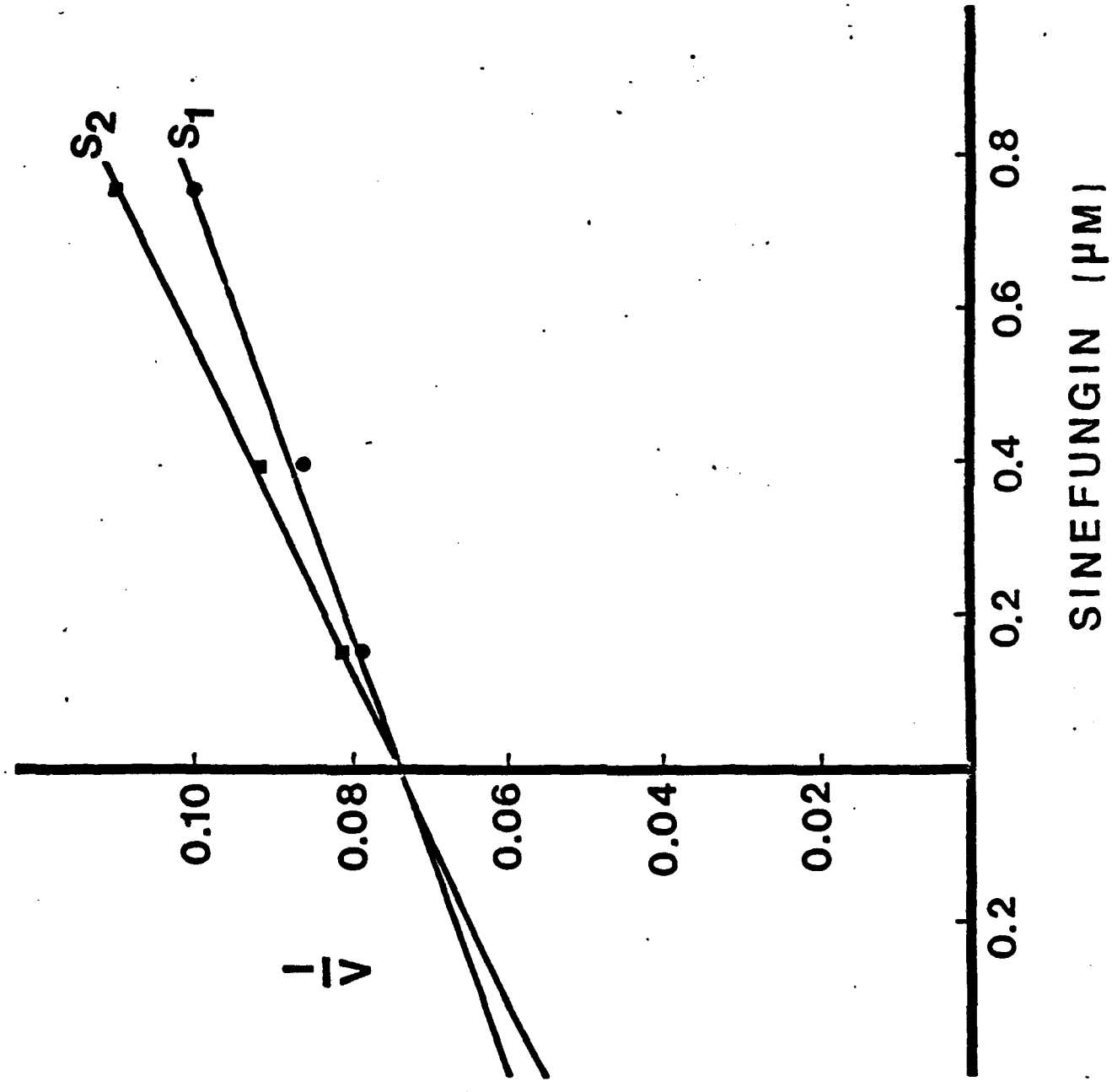
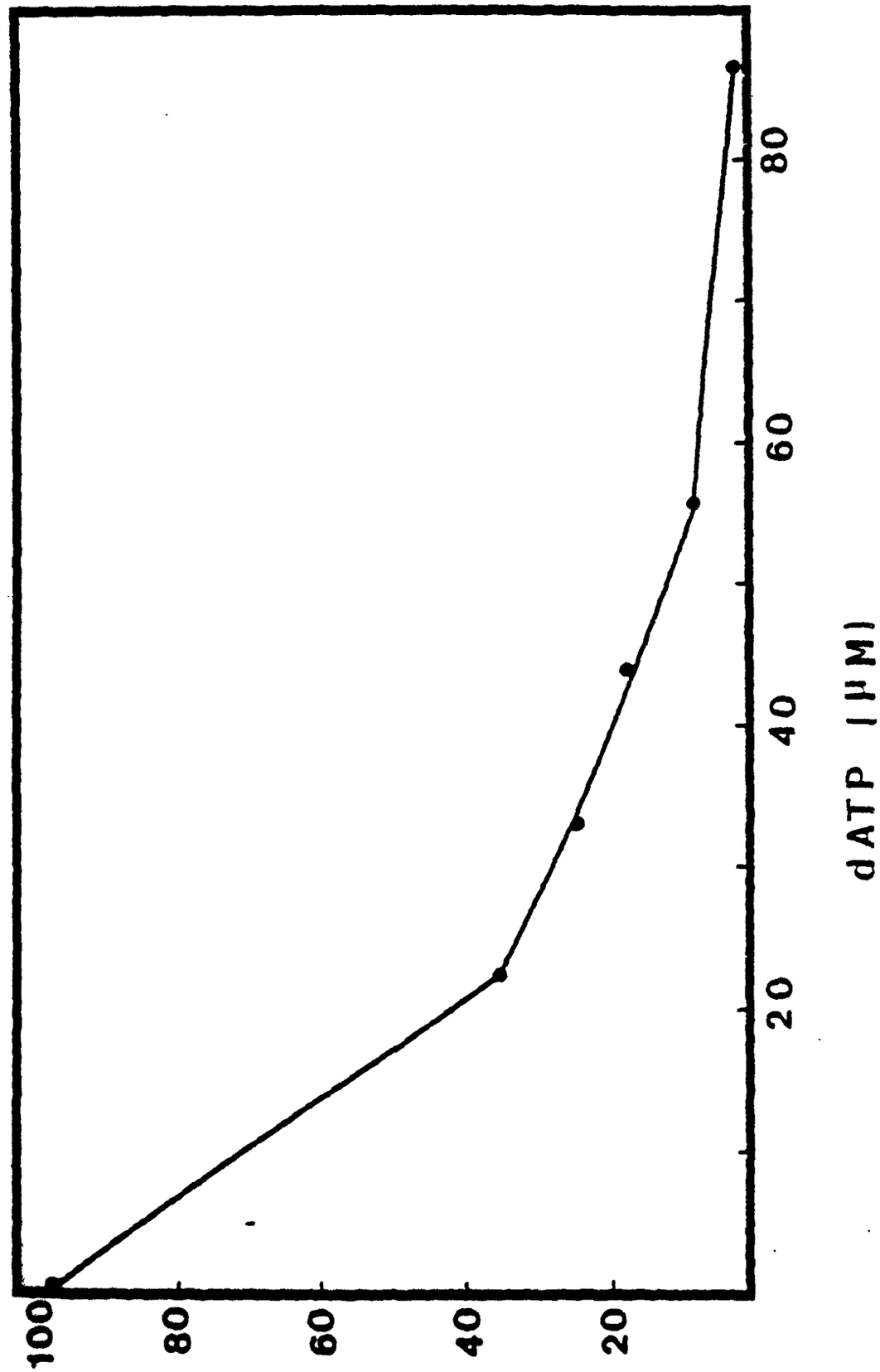


Fig. 3



Sinefungin 0.5 μM
Reversal of inhibition by dATP

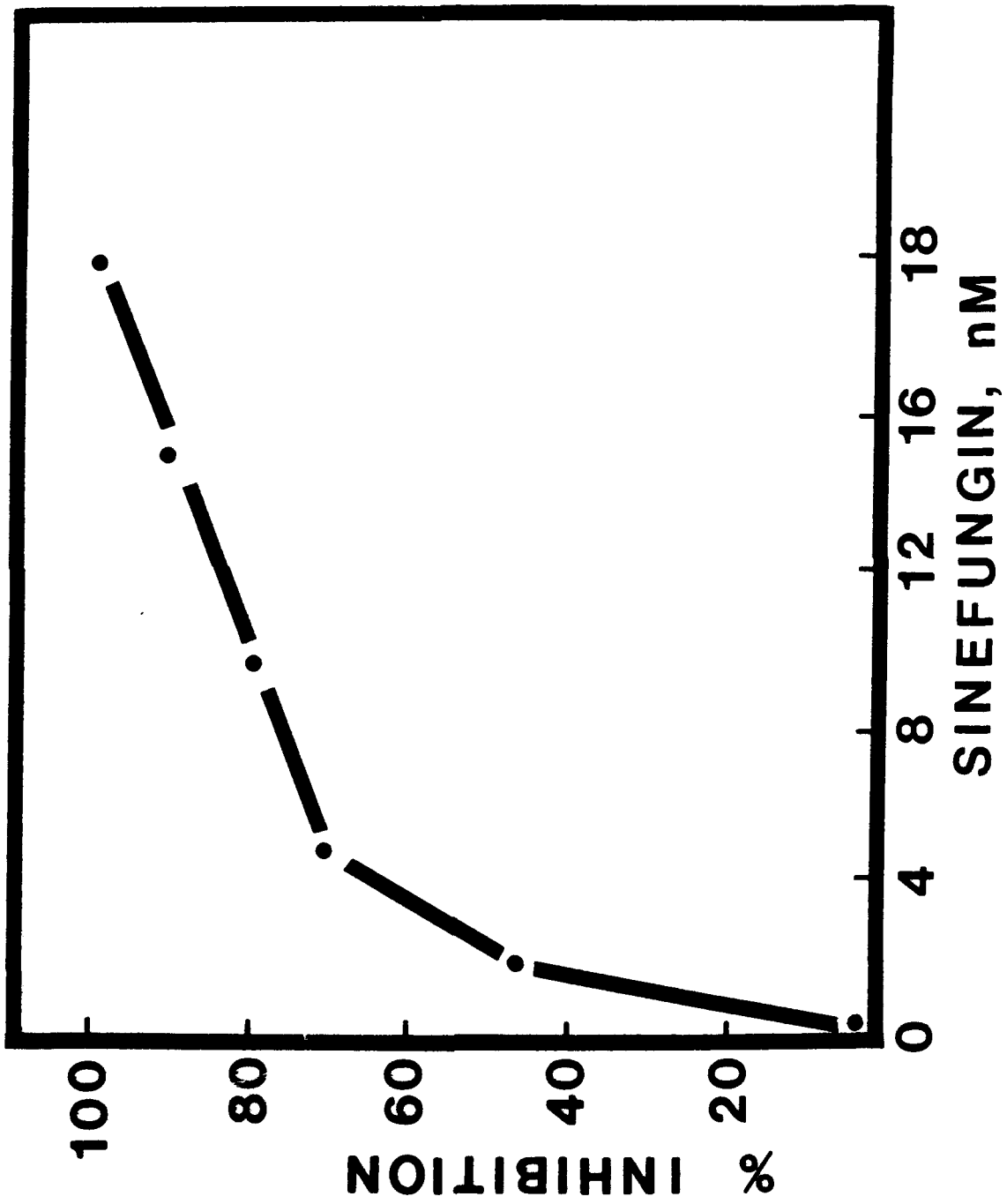
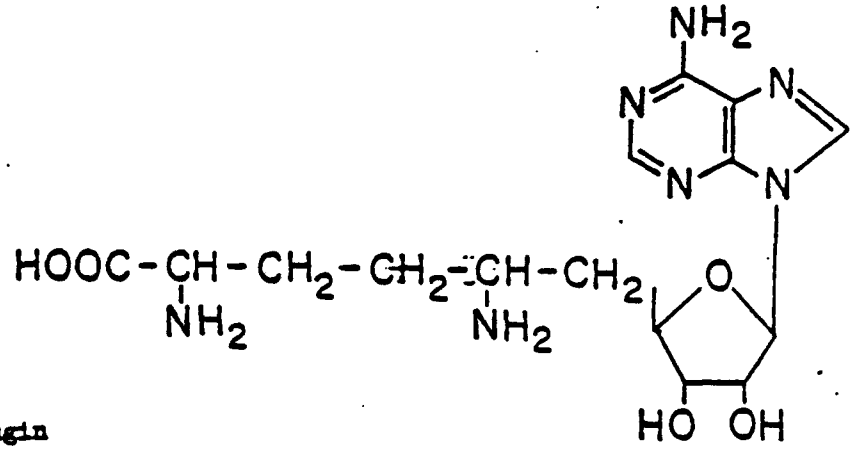
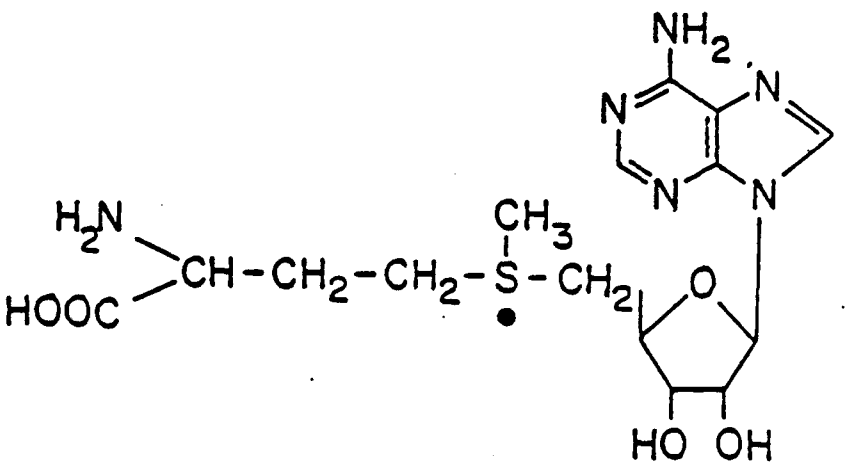


Figure 4. Growth Inhibition of L. mexicana By Sinefungin

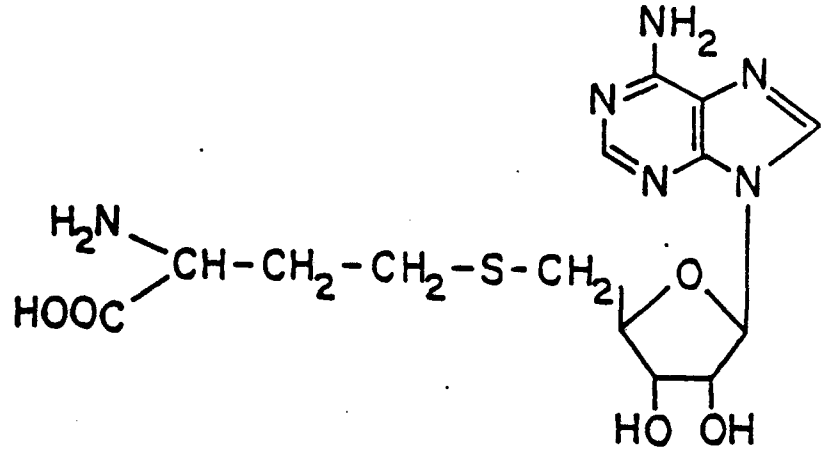
Fig. 5



Sinefungin



SAM



SAH

Table 16

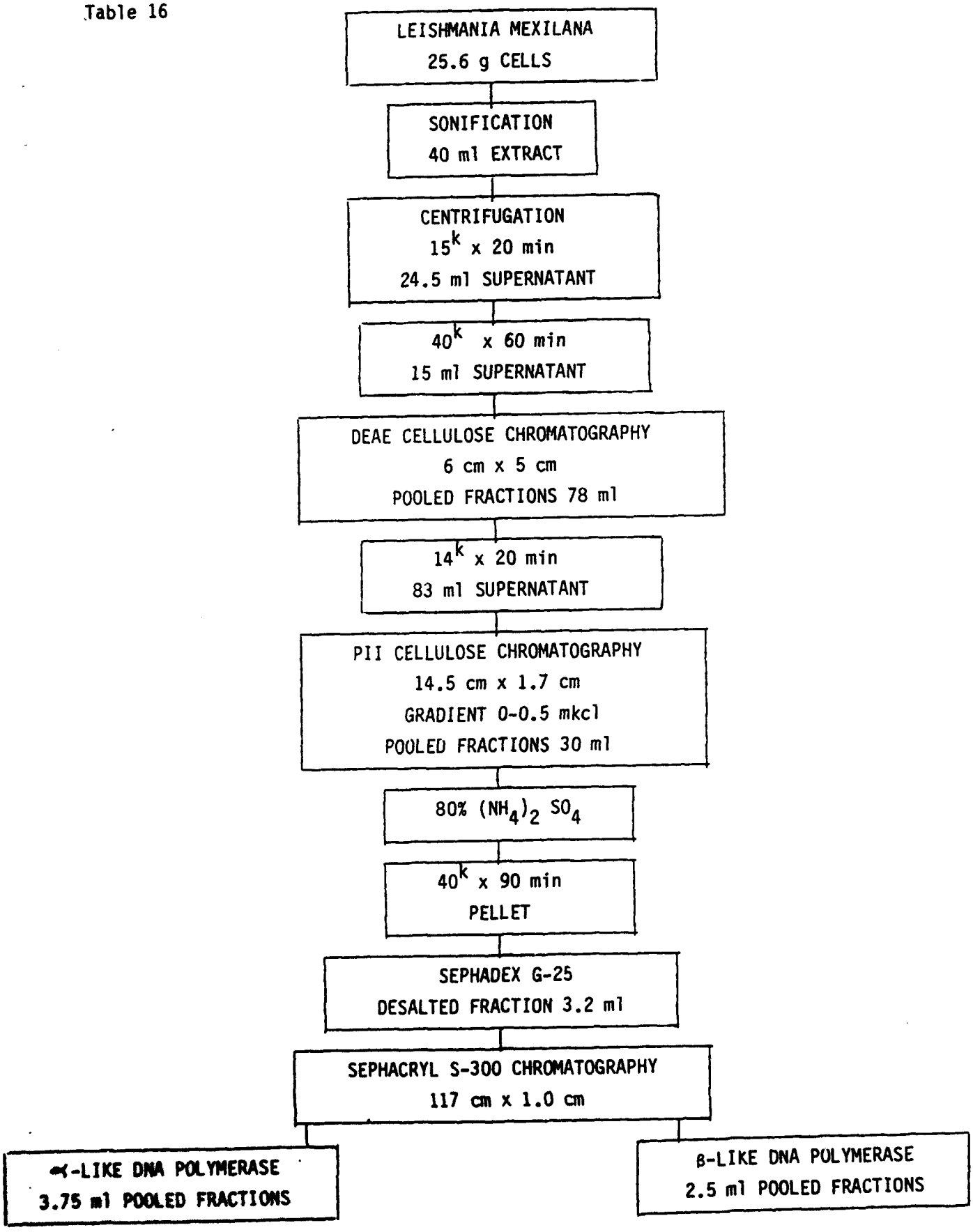

LEISHMANIA MEXILANA
25.6 g CELLS
SONIFICATION
40 ml EXTRACT
CENTRIFUGATION
15^k x 20 min
24.5 ml SUPERNATANT
40^k x 60 min
15 ml SUPERNATANT
DEAE CELLULOSE CHROMATOGRAPHY
6 cm x 5 cm
POOLED FRACTIONS 78 ml
14^k x 20 min
83 ml SUPERNATANT
PII CELLULOSE CHROMATOGRAPHY
14.5 cm x 1.7 cm
GRADIENT 0-0.5 mkcl
POOLED FRACTIONS 30 ml
80% (NH4)2 SO4
40^k x 90 min
PELLET
SEPHADEX G-25
DESALTED FRACTION 3.2 ml
SEPHACRYL S-300 CHROMATOGRAPHY
117 cm x 1.0 cm
α-LIKE DNA POLYMERASE
3.75 ml POOLED FRACTIONS
β-LIKE DNA POLYMERASE
2.5 ml POOLED FRACTIONS

figure 6. SEPARATION OF DNA POLYMERASE ALPHA AND BETA ON SEPHACRYL S300

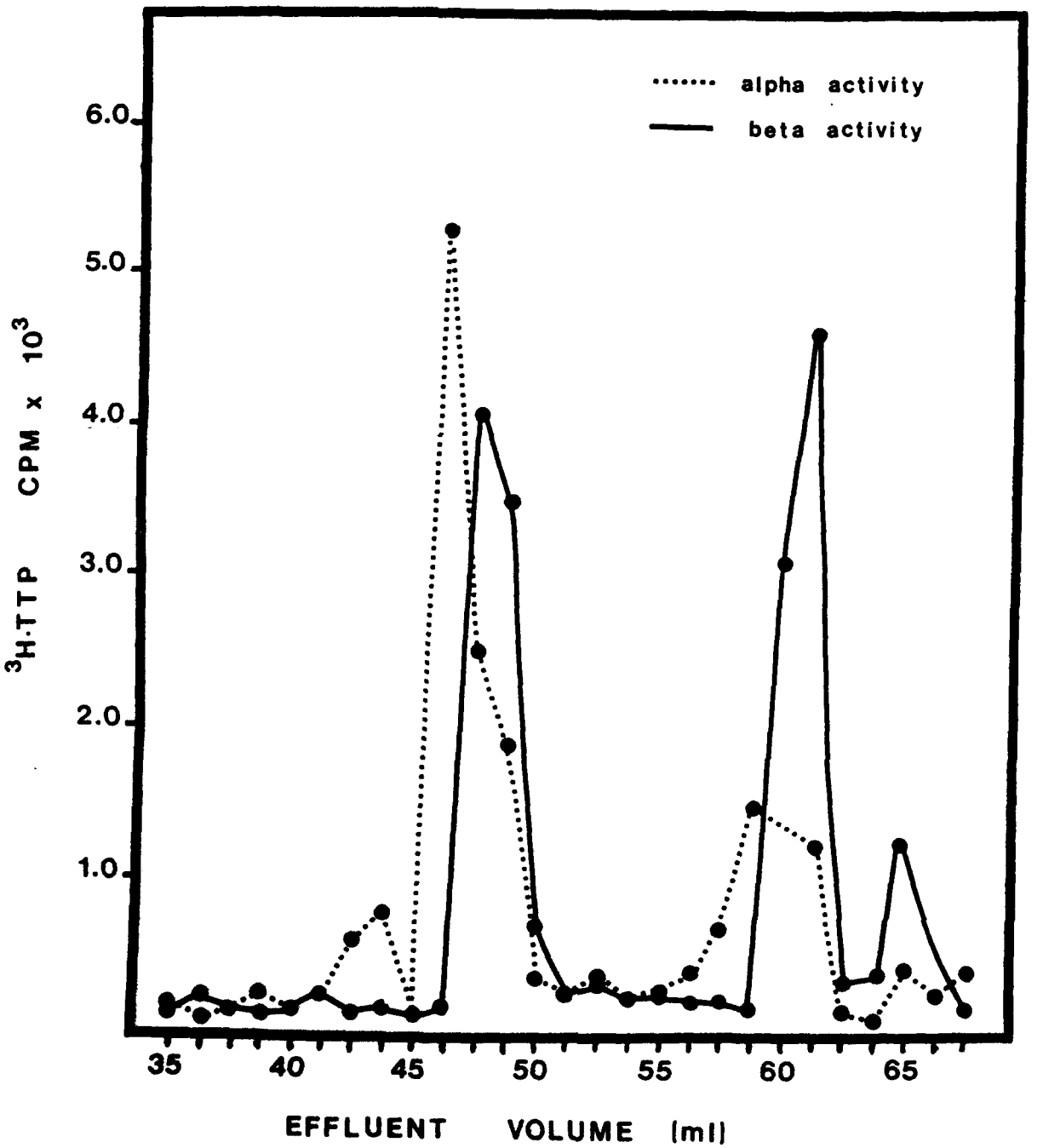


Fig. 7.

EFFECT OF ARACHIDONIC ACID ON DNA POLYMERASE FROM LEISHMANIA MEXICANA

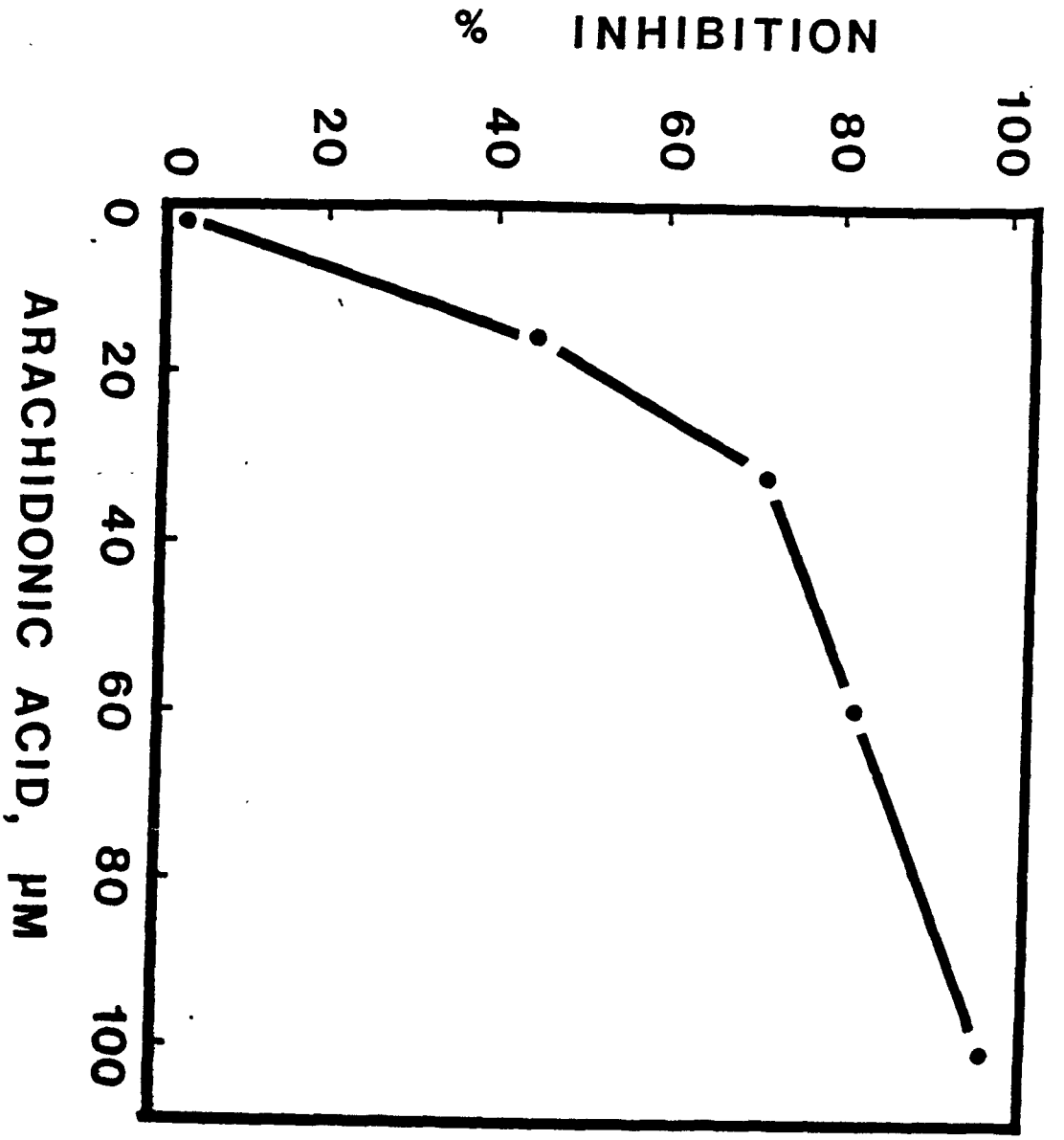
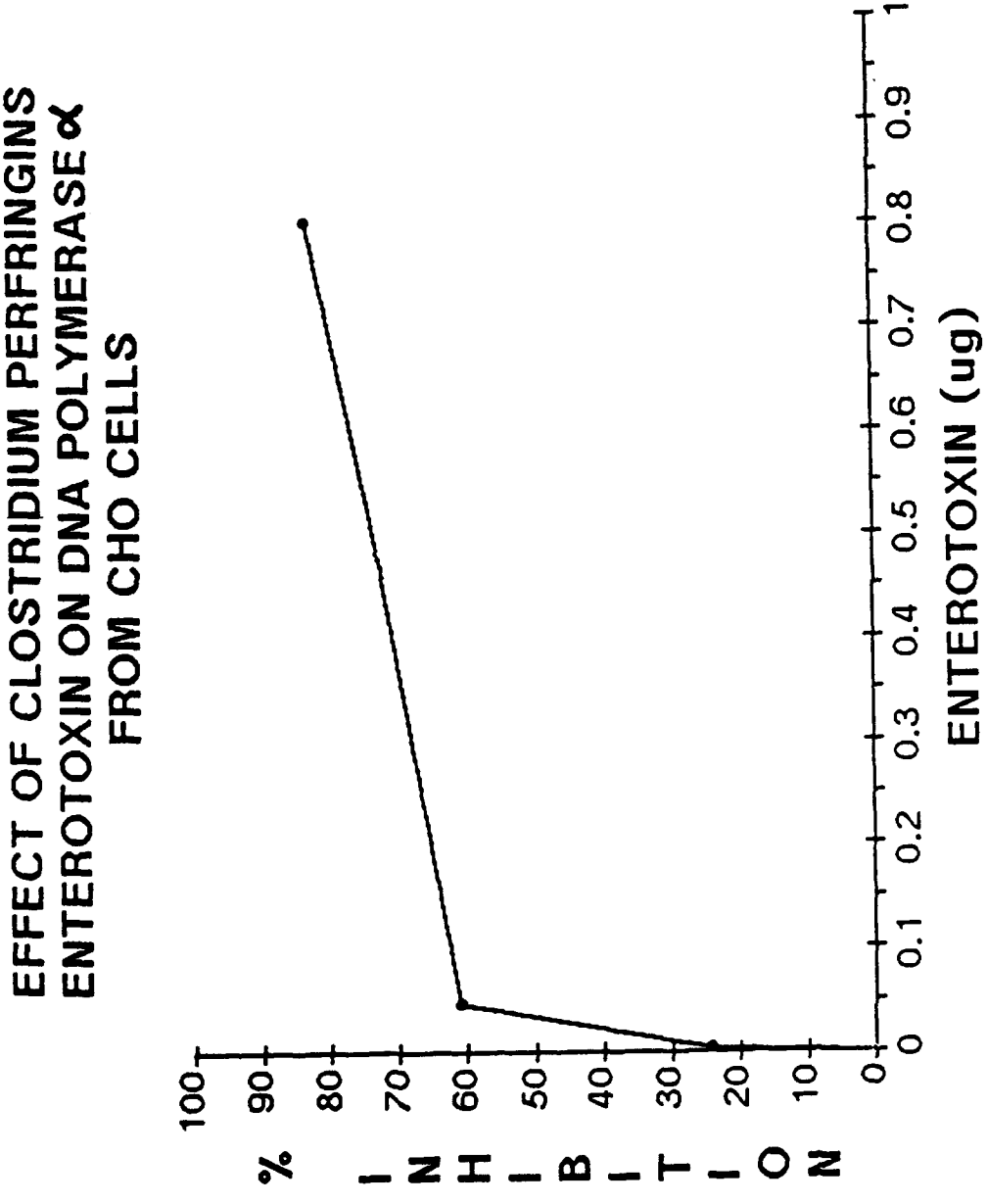


Fig. 8.


EFFECT OF CLOSTRIDIUM PERFRINGINS ENTEROTOXIN ON DNA POLYMERASE α FROM CHO CELLS
% INHIBITION
100
90
80
70
60
50
40
30
20
10
0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
ENTEROTOXIN (ug)

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